Eur. J. Biochem. 267, 1813–1818 (2000) © FEBS 2000

A second functional Δ5 fatty acid desaturase in the cellular slime mould *Dictyostelium discoideum*

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A cDNA with homology to fatty acid desaturases was selected by searching the cDNA data bank of *Dictyostelium discoideum* (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) with conserved histidine box motifs. Using this sequence, genomic DNA encoding the Δ5 desaturase was amplified from the genomic DNA of *D. discoideum*, and its desaturase activity was confirmed by the overexpression mutation in *D. discoideum* and the gain-of-function mutation in yeast. The cloned cDNA is 1565 nucleotides in length, and the deduced amino-acid sequence comprised 467 amino-acid residues containing an N-terminal cytochrome *b*5 domain that shared 43% identity with cytochrome *b*5 of *Oryza sativa*. The whole sequence was 42% identical to the Δ5 desaturase of *Mortierella alpina*. This desaturase is a novel member of the cytochrome *b*5-containing Δ5 fatty acid desaturase. As we have already reported one other Δ5 desaturase in *Dictyostelium*, this organism is the first to be confirmed as having two functional Δ5 fatty acid desaturase genes. The substrate specificities of the two functional Δ5 desaturases of *D. discoideum* were also examined.

Keywords: cDNA project; cytochrome *b*5; delta 5; desaturase; *Dictyostelium discoideum*.

Many unusual fatty acids have been found in cellular slime moulds, and unsaturation patterns of fatty acids in *Dictyostelium discoideum* have been reported [1–3], but only a few studies on fatty acid desaturases have been published [4]. Biochemical analysis of membrane-bound desaturases has been limited because they are difficult to purify because of their hydrophobic nature. Molecular genetic approaches, particularly the use of *Dictyostelium* mutants, have provided a significant amount of information on desaturation reactions.

Recently, genes encoding for Δ6 desaturases from borage, nematode and rat [5–7] and for Δ5 desaturase from *Caenorhabditis elegans*, *Mortiella alpina* and *D. discoideum* have been identified [4,8–11,]. Mutual comparisons of their deduced amino-acid sequences revealed the presence of a highly conserved haem-binding motif and a histidine box, $HX_{2(3)}$[XH]H, located in the same order.

The *Dictyostelium* cDNA genome project, in which we have participated, was initiated in Japan and almost a half (≈ 5000) of the total cDNAs expressed in the organism have already been identified as expressed sequence tags (ESTs). The determined ESTs have been deposited into the *Dictyostelium* cDNA database (Dicty_cDB, http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) [12]. We searched this *Dictyostelium* cDNA database with the histidine box motif, which is essential for desaturase activity, and we identified some clones. One of them, SSG614, has conserved histidine box motifs and a cytochrome *b*5 domain in the N-terminal region.

Using sequence information on the desaturases in eukaryotes, we identified the cDNA encoding functional Δ5 desaturase.

We have already reported on one other Δ5 desaturase in *Dictyostelium*; therefore, this is the first report to describe the presence of two cloned functional Δ5 fatty acid desaturase genes.

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Abbreviations: EST, expressed sequence tag; FAME, fatty acid methyl ester.

Note: the novel sequence data published here have been submitted to the DDBJ/EMBL/GeneBank sequence data bank with the accession number AB022097and the *Dictyostelium* cDNA data bank with the clone number SSG614 (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html)

(Received 1 November 1999, accepted 25 January 2000)

MATERIALS AND METHODS

Cell culture

Dictyostelium discoideum axenic strain Ax2, grown in HL-5 medium, was used as the wild-type strain. All of the transformants were grown under selection with the aminoglycoside antibiotic G418 at 10 $\mu g \cdot mL^{-1}$ in suspension [13].

Cloning and sequencing

The His-box motif, essential for desaturase activity, was searched for in the *Dictyostelium* cDNA database; the clone SSG614 with conserved His-boxes was found. The general motif $HX_{2(3)}$[XH]H was used as query sequence and a pattern search program was used to find the putative desaturase clones (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html). To isolate the genomic DNA of this clone, PCR amplification of Ax2 genomic DNA was performed with the forward primer 5′-*AAGCTT*ATGATGGAAACAAATAATG-3′ (annealing to the initiating methionine, indicated by bold type) and the reverse primer, 5′-*AAGCTT*AATACAGTTTGATTAATCG-3′ (annealing to the compliment of the stop codon, indicated by bold type). Each primer contained a *Hin*dIII restriction site (italic). The resulting 1.6-kb product was ligated into the pT7Blue T-vector (Novagen) and transformed into competent

DH5-alpha *Escherichia coli* cells. After purification of the plasmids, the whole nucleotide sequence was determined by the dideoxy chain termination method [14] using an Applied Biosystems PRISM Ready Reaction Dye Terminator Cycle Sequence Kit. The sequencing reactions were run on a 377XL automated DNA sequencer (Perkin-Elmer, Division of Applied Biosystems).

Functional analysis

Dictyostelium transformation. The primers described above were used to amplify the SSG614 coding region and flank it with the *Hin*dIII restriction site (underlined). The amplified PCR product was ligated in to the *Hin*dIII site of pDEX-RH (a gift from J. Faix) to generate an overexpression construct. This vector was derived from pDEX-H [15] by introducing an *Eco*RI restriction site into the cDNA expression cassette, downstream of the actin 15 promoter [16]. DNA sequencing was used to confirm that the entire sequence of the insert and the flanking sequences was as expected. The construct was purified with a Plasmid Maxi Kit (Qiagen) and *D. discoideum* cells were transformed according to the method described previously [17–19].

Yeast transformation. The yeast vector pYES2 (Invitrogen) was digested with *Hin*dIII and the PCR fragment, which was amplified with the above-mentioned primers, and ligated with pYES2 to generate a transformation construct. After confirmation by DNA sequencing analysis, the plasmid was transformed into *Saccharomyces cerevisiae* strain INVSc1(Invitrogen) by the lithium acetate method [20]. The recombinant yeast cells were selected on a uracil-deficient medium, and the transgene was induced by the addition of galactose to 2% (w/v) and further culture for 16 h at 30 °C in the presence of 0.5% NP-40 with or without 0.5 mM C18:1(11). In order to determine the substrate specificity, the Δ5 desaturase activity in recombinant yeast was expressed as the percentage Δ5 desaturated fatty acids of the total amount of substrate.

Fatty acid analysis

Total lipids were extracted by the method of Bligh and Dyer [21] from wet cells in the vegetative growth phase. Alkaline methanolysis of the total lipids released ester-linked fatty acids as methyl esters (FAMEs). Total FAMEs were analysed by GC (model GC-8A; Shimadzu, Japan) using a flame-ionization detector and a capillary column (CPS-1; 50 m × 0.25 mm, i.d.; Quadrex, New Haven, Connecticut, USA). The separated FAMEs were identified by comparing their retention times with those of authentic standards on the same GC, and they were quantified with a data analyser (C-R6A chromatopac; Shimadzu, Japan) as described previously [22]. The position of a double bond in FAME was determined by the pyrrolidine method and by the I_2-catalysed reaction for the formation of the adducts with dimethyldisulfide as described previously [4,22,23].

RESULTS AND DISCUSSION

Amino-acid sequence of a *Dictyostelium* desaturase and comparison with other desaturases

Comparison of the deduced amino-acid sequence of the fatty acid desaturase and related proteins from mammals, fungi, higher plants and cyanobacteria revealed highly conserved regions that contain histidine residues [24]. As the first stage of the Japanese cDNA genome project on the cellular slime mould *D. discoideum* has been finished and almost one-half (≈ 5000) of the entire *Dictyostelium* cDNAs have been identified as nonredundant ESTs, we searched for the histidine box motif essential to desaturase activity in the cDNA database and identified one clone, SSB813, as Δ5 desaturase (Dd des5-1) [4]. In the course of the characterization of this desaturase, we noted the presence of a related sequence in the database (clone SSG614). This clone encodes an open reading frame (ORF) of 467 amino-acids (Fig. 1). A closer examination of this ORF revealed a number of similarities with Dd des5-1, in that it contained an N-terminal cytochrome *b*5 domain, as defined by the diagnostic HPGG motif (circled in Fig. 1), as well as three histidine boxes. These boxes have the general sequence of $HX_{2(3)}$[XH]H (underlined in Fig. 1). The third histidine box contains a histidine to glutamine substitution, which is also evident in the Δ5 desaturases of slime mould, fungi and nematode [4,8–11]. The distance between the first and the second boxes was 31 amino acids, compared with 31 or 32 amino acids in other desaturases. The spacing between the second and the third histidine box is 183 amino acids for this desaturase. This distance is less well-conserved among the membrane-bound desaturases and varies from 132 to 177 amino acids. As the spacing between those boxes in the *Dictyostelium* Δ5 desaturase Dd des5-1 is 177 amino acids, it is likely that the length of the spacing is rather long in Δ5 desaturase compared with that of other desaturases. The importance of these histidine boxes in catalysis has been demonstrated by site-directed mutagenesis of Δ9 desaturase from the rat and Δ12-desaturase of *Synechocystis* [24–26]. Fig. 2 shows the alignment of the deduced amino-acid sequence of SSG614 (indicated by Dd des5-2) with other Δ5 desaturases. The whole sequence was 42% and 66% identical to the Δ5 desaturase of *M. alpina* and *D. discoideum* (Dd des5-1), respectively. The alignment of the N-terminal sequences (1–100) of SSG614 with those of microsomal cytochrome *b*5 proteins and nitrate reductases from various higher plants revealed that the sequence identity is ≈ 40–45%. Further evidence of similarity is the presence of eight invariant residues (circled in Fig. 1) of the cytochrome *b*5 class of proteins

Table 1. Fatty acid compositions of the empty vector control and pDEX-RH/614 transformed cells. Each value is the mean ± SD of three independent experiments. Some minor components (less than 0.1%) were omitted. Tr, trace (< 0.1%).

Fatty acid	Empty vector (%)	pDEX-RH/614(%)
16:0	5.6 ± 0.1	8.1 ± 1.6
16:1(5)	0.7 ± 0.1	4.9 ± 1.7
16:1(9)	6.2 ± 0.7	4.6 ± 0.9
16:2(5,9)	2.2 ± 0.5	1.7 ± 0.2
17:0	0.4 ± 0.1	0.7 ± 0.2
17:1(5)	Tr	0.7 ± 0.3
17:1(9)	0.3 ± 0.1	0.2 ± 0.2
17:2(5,9)	1.2 ± 0.2	2.0 ± 0
18:0	1.0 ± 0.3	1.1 ± 0.5
18:1(5)	0.3 ± 0.2	1.3 ± 0.4
18:1(9)	2.9 ± 0.1	1.1 ± 0.3
18:1(11)	27.6 ± 1.4	14.6 ± 3.4
18:2(5,9)	15.1 ± 2.1	11.7 ± 1.6
18:2(5,11)	34.8 ± 1.6	46.5 ± 0.5
18:2(7,11)	1.6 ± 0.6	0.7 ± 0.2

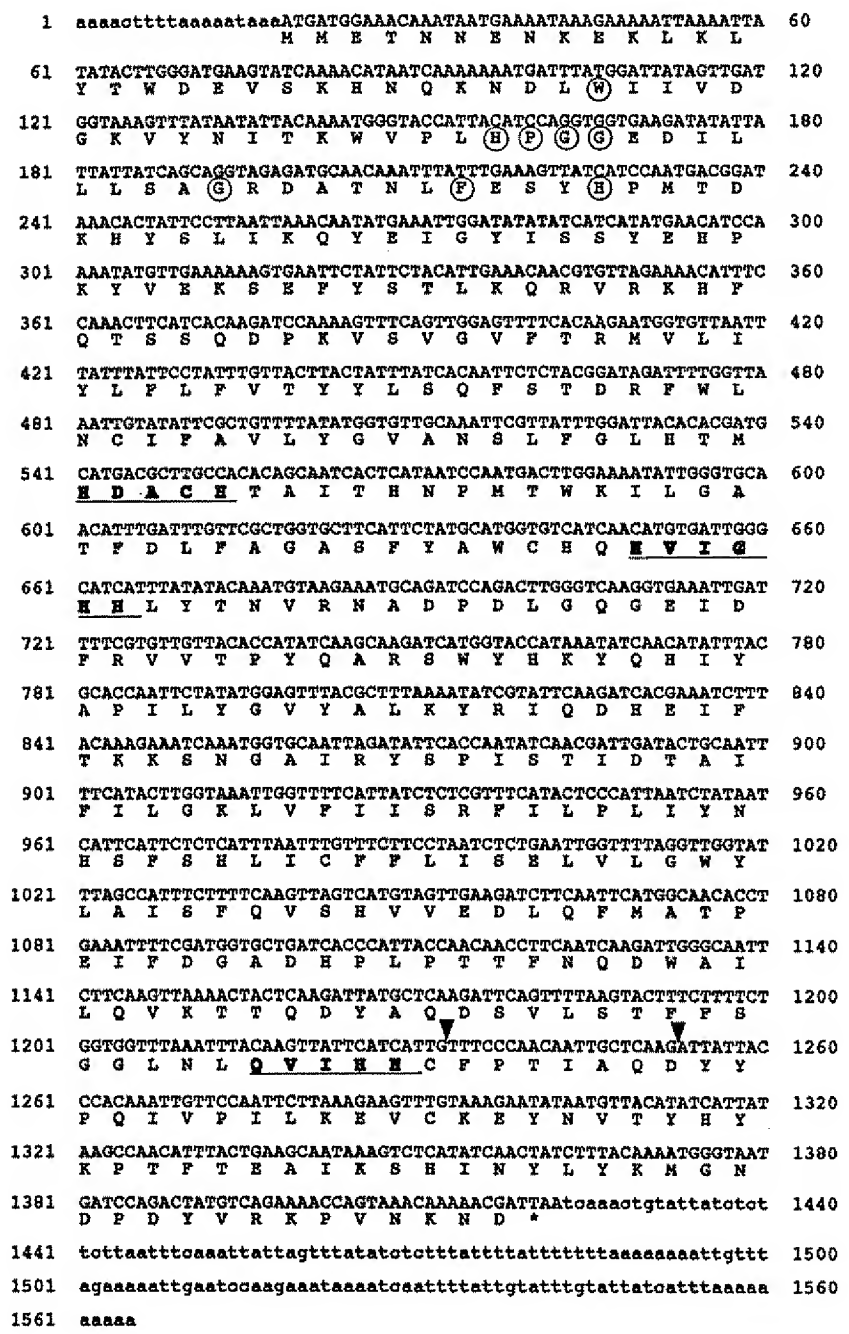

Fig. 1. Deduced amino-acid sequence of the cDNA from *D. discoideum*. The three conserved histidine regions are underlined. The eight invariant amino-acid residues (circled) are known to be essential for the catalytic activity of cytochrome *b5* in stearoyl-CoA desaturase [24]. The arrowheads indicate the positions of introns. The sequence data published here have been submitted to the DDBJ/EMBL/GeneBank sequence data bank an assigned the accession number AB022097.

identified by Lederer [27]. The identity is particularly high in the regions previously identified as essential for cytochrome *b5* function, including the HPGG motif in the haem-binding region (Fig. 2). A haem-containing electron donor is required for fatty acid desaturation and cytochrome *b5* fulfils this function for the membrane-bound desaturases [28–31]. The whole sequence can be considered as coding for a fusion protein consisting of an N-terminal cytochrome *b5* and a desaturase.

The predicted hydrophobicity plot for the desaturase from clone SSG614 also revealed a profile characteristic of a fatty acid desaturase, with the histidine boxes located in hydrophilic areas and separated by hydrophobic regions (data not shown) [24,26,31].

To examine the structure of genomic DNA (the presence of introns), we amplified Ax2 genomic DNA with the primers described in Materials and methods. The genomic DNA contained two stretches as introns, one of 79 nt and the other of 85 nt (indicated by arrowheads in Fig. 1). The genomic sequence of Dd des5-1 also contained two stretches of introns. The second intron of Dd des5-1 was located at a position similar to the first intron within this Δ5 desaturase sequence.

Both introns of this desaturase have the typical characteristics seen in *Dictyostelium* introns consisting of oligo(dT) and oligo(dA) stretches flanked by consensus 5′ and 3′ splice sites GTAAGT and AG, respectively. Furthermore no frameshift occurs when this region is spliced out.

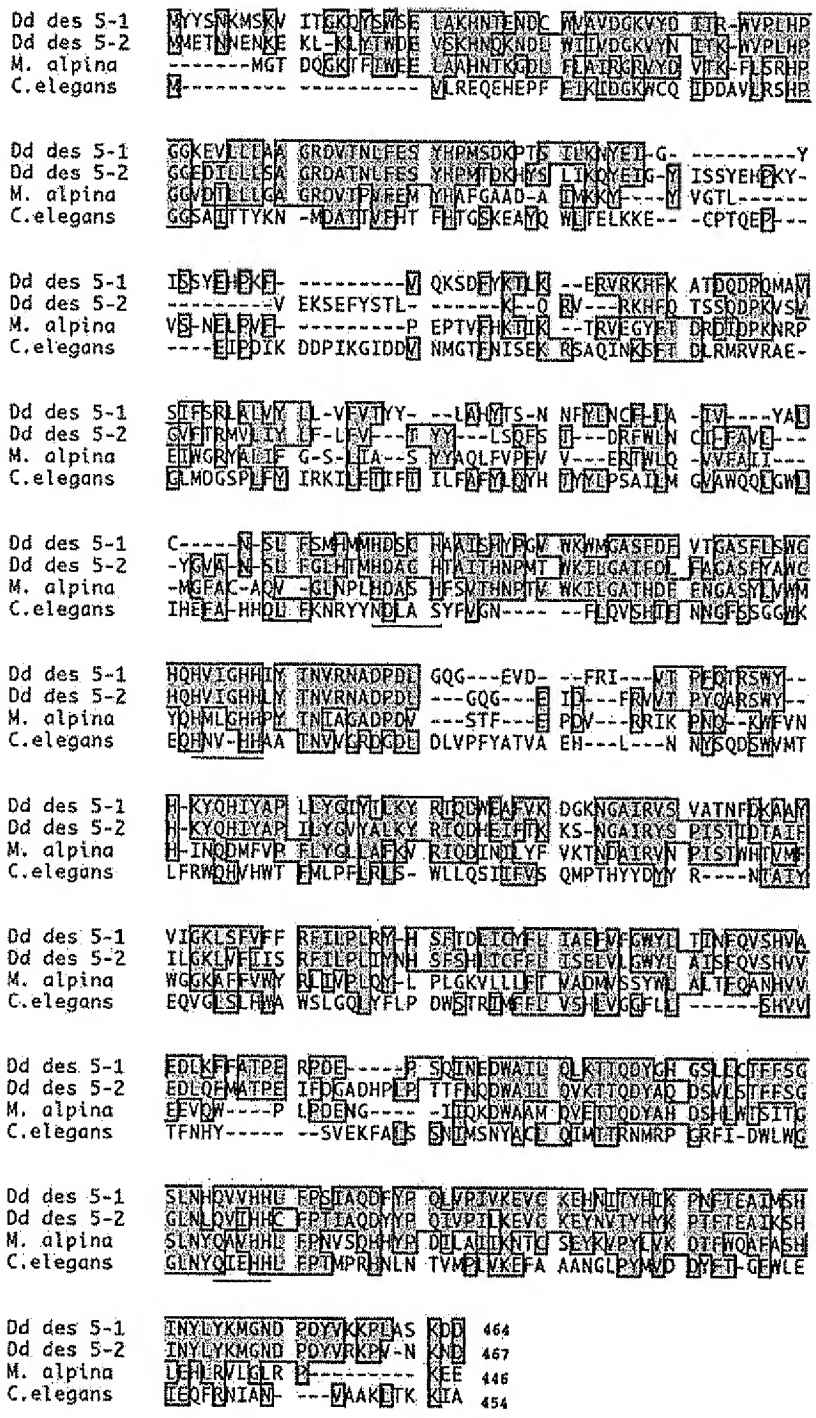

Fig. 2. Comparison between Δ5 desaturases of *D. discoideum*, *M. alpina* and *C. elegans*. The deduced amino-acid sequence of the coding region of SSG614 (indicated by Dd des5-2) is aligned with those of the Dd des5-1, *M. alpina*, *C. elegans* Δ5 desaturase genes. Identical residues are shaded and the conserved histidine boxes are underlined.

Functional analysis

To analyse the function of cDNA corresponding to SSG614, a cDNA fragment was inserted into the *Dictyostelium* expression vector pDEX-RH downstream of the actin 15 promoter (pDEX-RH/614). *Dictyostelium* cells were transformed with this construct to express the putative desaturase gene constitutively. Two independent transformants were isolated and total lipids were extracted from them, empty vector control and wild-type (nontransformed) cells, and they were transmetylated. The resulting FAMEs were prepared from the total lipid extracts and analysed by GC. The fatty acid compositions of wild-type cells and empty vector transformants were essentially the same.

Table 1 shows the fatty acid compositions of empty vector control and pDEX-RH/614-transformed cells. C16:1(5) fatty acid accounted for 0.7% of total fatty acids in empty vector control cells but ≈ 4.9% of total fatty acids in pDEX-RH/614-transformed cells. The amount of C18:2(5,11) was also increased with accompanying decrease in C18:1(11) in the pDEX-RH/614-transformed cells. This acid accounted for 34.8% of total fatty

Table 2. Substrate specificities of Dd des5-1 and Dd des5-2 in the transgenic yeast. Each value is the mean ± SD from three independent experiments. ND, not detected.

Substrate	Dd des 5-1 Substrate converted (%)	Dd des 5-2 Substrate converted (%)
16:0	13.0 ± 0.5	ND
16:1(9)	37.9 ± 0.4	ND
18:0	ND	ND
18:1(9)	45.3 ± 0.4	6.1 ± 1.0
18:1(11)	34.1 ± 0.9	12.1 ± 1.2

acids in the control, and 46.5% in the transformed cells. On the other hand, the percentage of C18:2(5,9) is low with respect to that of empty vector control, despite the fact that the amount of C18:1(9) is also decreased in the transformed cells. This may be caused by the substrate specificity of this desaturase.

The increase in these fatty acids would be due to the expression of the introduced gene in the pDEX-RH/614-transformed cells but not in the empty vector control cells and nontransformed cells. This suggests that the cDNA encodes Δ5 desaturase. To confirm this, the complete coding region of this cDNA was inserted into the yeast expression vector pYES2 downstream of the GAL1 promoter (pYES2/614). This construct was transferred to *S. cerevisiae*. Cells were cultured overnight in a medium containing glucose as a carbon source. Expression of the SSG614 coding region from the GAL1 promoter of the vector was induced by changing the medium to one containing 2% galactose and further cultivation for 16 h at 30 °C. Figure 3 shows the results of GC analysis of FAMEs of pYES2/614 transformed cells and the empty vector control. An additional two peaks are apparent in the trace obtained from the induced pYES2/614-transformed cells but not from the induced empty vector control. These peaks are also absent from the nontransformed cells. The retention times of these additional peaks were identical to those of C18:2(5,9) and C18:2(5,11), respectively. These peaks were analysed further by GC/MS and identified as C18:2(5,9) and C18:2(5,11), respectively (data not shown).

The results show that the yeast cells transformed with pYES2 containing the SSG614 coding region had gained the function of Δ5 desaturation and that the product of this gene acted on the monounsaturated substrates to give C18:2. These results demonstrate that the cDNA of SSG614 encodes a Δ5 desaturase of the cellular slime mould *D. discoideum*. We named this Δ5 desaturase Dd des 5-2.

Substrate specificity of two functional Δ5 desaturases in *D. discoideum*

We identified a second functional Δ5 desaturase gene in *D. discoideum*. This indicates that *D. discoideum* has two functional Δ5 desaturase genes in the genome. The amino-acid sequences encoded by two genes are 66% identical to each other, and each contains an N-terminal cytochrome *b5* and three histidine boxes. Both genes contain a histidine to glutamine variant that has so far been shown to be unique to the desaturases involved in double-bond insertion at the carbon below position 9. Despite these similarities, these two desaturases show different substrate specificity. When overexpressed in

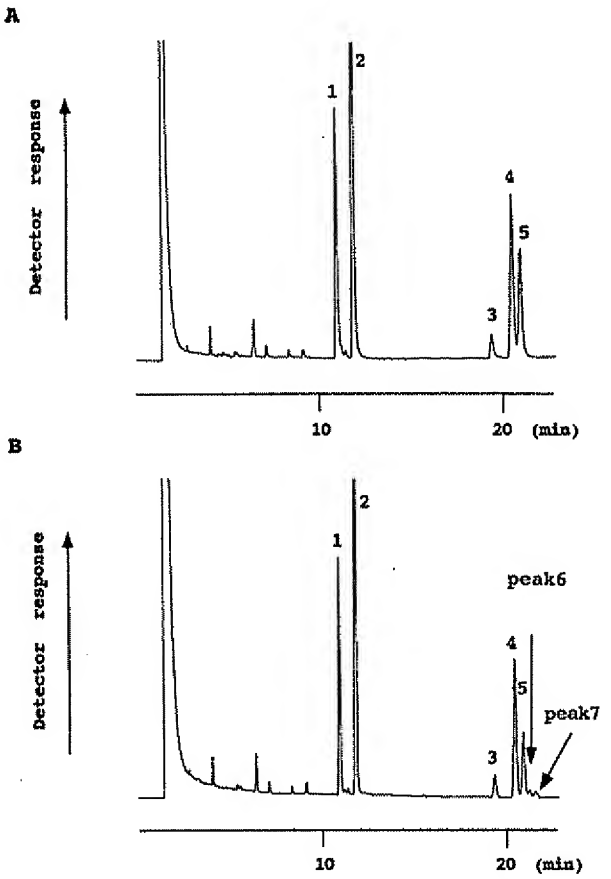

Fig. 3. Identification of Δ5 desaturated fatty acids in transgenic yeast. FAMEs of total lipids of *S. cerevisiae* grown under induction conditions with galactose in the presence of C18:1(11) were analysed by GC. (A) FAMEs extracted from yeast transformed with an empty vector, pYES2. (B) FAMEs extracted from yeast transformed with pYES2/813. The common peaks were identified as C16:0 (peak 1), C16:1(9) (peak 2), C18:0 (peak 3) C18:1(9) (peak 4) and C18:1(11) (peak 5). Peaks 6 and 7 are additional peaks, and they were analysed by GC/MS and identified as C18:2(5,9) (peak 6) and C18:2(5,11) (peak 7).

S. cerevisiae, Dd des 5-2 specifically acts on C18 monoenoic acids. In contrast, Dd des 5-1 acts on C16 and C18 fatty acids under these conditions (Table 2). As for Dd des 5-2, 12.1% of C18:1(11) was converted to C18:2(5,11), while only 6.1% of C18:1(9) was converted to C18:2(5,9) in our experiment. This result is consistent with the fatty acid composition of the pDEX-RH/614-overexpressing mutant, although C16:1(5) could not be detected in transformed yeast, because the major fatty acid in the pDEX-RH/614-overexpressing mutant is C18:2(5,11).

In order to determine the exact substrate specificities, gene knockout experiments on each desaturase should be performed in a further study.

ACKNOWLEDGEMENTS

We thank the members of the Japanese cDNA project on *Dictyostelium discoideum* for providing the clone SSG614. We acknowledge the technical assistance with GC/MS of E. Fukusi, Department of Agriculture, Hokkaido University. This work was supported by a grant from Kurata Foundation to T. S. and in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 08283105).

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2007 WL 1766992 (Bd.Pat.App. & Interf.)

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Decided: June 15, 2007

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Before DONALD E. ADAMS, DEMETRA J. MILLS, and RICHARD M. LEBOVITZ
Administrative Patent Judges
LEBOVITZ
Administrative Patent Judge

DECISION ON APPEAL

This is a decision on appeal from the final rejection of claims 17-19, 21-24, 26-28, 30-38, and 41-46. We have jurisdiction under 35 U.S.C. § 6(b). We reverse the rejections under 35 U.S.C. § 112, first and second paragraphs and § 103, but affirm the rejection for nonstatutory obvious-type double-patenting.

STATEMENT OF CASE

> Arthrobacter aurescens DSM 3747 is one of the few isolated microorganisms capable of converting 5-monosubstituted hydantoins to L-amino acids. The disadvantage of using A. aurescens cells as [a] biocatalyst is the low enzyme activity. Especially the L-N-carbamoylase is the bottleneck for most substrates leading to an increase of the intermediate L-N-carbamoyl amino acid in the cell, which is not further converted to the corresponding amino acid.

(Specification 1: 28 to 2: 3.)

The asymmetric bio-conversion to either L- or D-amino acids involves three enzymes: hydantoinase, hydantoin racemase, and D- or L-specific carbamoylase (Specification 1: 19-27; 2: 8-16). The claimed invention is directed to microorganisms ("whole cell catalysts") transformed with DNAs coding for hydantoinase, hydantoin racemase, and carbamoylase, and methods of using the microorganisms to produce enantiomerically enriched amino acids.

> Using whole cell catalysts comprising cloned genes encoding for a hydantoinase, for a hydantoin racemase and a D- or L-specific carbamoylase for the conversion of 5-monosubstituted hydantoins to L- or D-amino acids results in a fast and complete conversion of racemic mixtures of hydantoins to the corresponding L- or D-amino acids on industrial scale. This significantly reduces the production costs due to a reduction of fermentation and purification costs because all enzymes are produced in one strain.

(Specification 3: 1-9.)

Claims 17-19, 21-24, 26-28, 30-38, and 41-46 are on appeal (Br. 4). Claims 39 and 40 have been allowed (Br. 4). The appealed claims stand rejected under 35 U.S.C. § 112, first and second paragraphs, 35 U.S.C. § 103(a), and nonstatutory obvious-type double-patenting (Br. 9).

The Examiner relies on the following as evidence of unpatentability:

Wagner US 5,827,717 Oct. 27, 1998

*2 Van de Loo, *Proc. Natl. Acad. Sci.*, 92: 6743-6747 (1995).

Broun, *Science*, 282: 1315-1327 (1998).

Bork, *Genome Research*, 10:498-400 (2000).

We select claim 17, the broadest and only independent claim on appeal, as representative:

17. A microorganism which

(A) is transformed with DNAs encoding (i) a hydantoinase, (ii) a hydantoin racemase, and (iii) a D- or L-specific carbamoylase, and

(B) converts 5-monosubstituted hydantoins to L- or D-amino acids,

wherein the DNAs encoding the hydantoinase, the hydantoin racemase, and the D- or L-specific carbamoylase are overexpressed in the microorganism

> according to the turnover rates of the respective enzymes to reduce the accumulation of intermediates in the conversion of the 5-monosubstituted hydantoins to L- or D-amino acids.

DISCUSSION

Rejection under § 112, second paragraph

Claims 17-19, 21-24, 26-28, 30-38, and 42-46 stand rejected under 35 U.S.C. § 112, second paragraph, as indefinite (Answer 3; Br. 9).

Claim 17 is directed to a microorganism transformed with DNAs encoding 1) hydantoinase, 2) hydantoin racemase, and 3) D- or L-specific carbamoylase. The DNAs are

> overexpressed in the microorganism according to the turnover rates of the respective enzymes to reduce the accumulation of intermediates in the conversion of the 5-monosubstituted hydantoins to L- or D-amino acids.

The Examiner states the claimed phrase is indefinite because "there are many turnover rates for an enzyme" and "it is not clear which 'turnover rates' are being referred to or how they relate to overexpression" (Answer 3-4).

Claims are interpreted as they would be understood by one of ordinary skill in the art "taking into account whatever enlightenment by way of definitions or otherwise that may be afforded by the written description contained in the applicant's specification." *In re Morris*, 127 F.3d 1048, 1054 (Fed. Cir. 1997).

According to the Specification, enzymes "naturally possess different turnover rates"[FN1] (Specification 3: 23-24). When the turnover "rates of co-working enzymes are not in line ... [,] intermediates accumulate ... inside the cell" (Specification 3: 24-26). Overexpression can also lead to "the formation of inclusion bodies ... which is unfavourable for a well balanced coexpression of all the three enzymes" (Specification 3: 26-30). "Therefore, various attempts to 'fine tune' the expression of these genes have been made. This can be done advantageously by overexpressing the hydantoinase genes in question according to their turnover rates" (Specification 3: 30-33).

Several different approaches are disclosed "[t]o adopt the turnover rate of all enzymes expressed in the whole cell" (Specification 5: 23-25), including by the use of different promoters, mutant enzymes, enzymes from different sources, and replicons (e.g., plasmids) with different copy numbers (Specification 5: 26 to 6: 29). Since "turnover rate" refers to the speed at which enzymes process their substrates, the amount of enzyme expressed in the cell determines how much substrate is processed in a given time period.

*3 "The test for definiteness is whether one skilled in the art would understand the bounds of the claim when read in light of the specification. If the claims read in light of the specification reasonably apprise those skilled in the art of the scope of the invention, § 112 demands no more." *Miles Laboratories, Inc. v. Shandon, Inc.*, 997 F2d 870, 875, 27 USPQ2d 1123, 1126 (Fed. Cir. 1993) (citations omitted).

In our opinion, the skilled worker would understand, in the context of the Specification, "the DNAs" are "overexpressed in the microorganism according to the turnover rates of the respective enzymes" means that the expression of the three different synthetic enzymes is adjusted to avoid accumulation of intermediates.

Fig. 5 shows that adjusting the expression of the enzymes according to their turnover rate avoids the accumulation of intermediate ("CaTrp") when the substrate ("IMH") is converted to the final product ("Trp") (Specification 7: 24-30). In contrast, Fig. 6, in which the enzyme expression levels differ from those in the experiment illustrated in Fig. 5 (Specification 10 (Table 1)), shows an experiment which results in the accumulation of the intermediate (CaTrp) (Specification 7: 31 to 8: 3).

Because the Specification reasonably apprises those skilled in the art of the scope of the claimed invention, we conclude that the claims are in conformance with § 112, second paragraph. We reverse the rejection of claims 17-19, 21-24, 26-28, 30-38, and 42-46.

Rejection under § 112, first paragraph, for lack of written description

Claims 17-19, 21-24, 26-28, 30-38, 42, 45, and 46 stand rejected under 35 U.S.C. § 112, first paragraph, as lacking an adequate written description of the claimed invention (Answer 4; Br. 9). The Examiner states that the claims are directed to "a microorganism transformed with a genus of DNAs encoding hydantoinases, hydantoin racemases, and/or carbamoylases, wherein all the DNAs can have any structure" (Answer 4). Relying on *University of California v. Eli Lilly and Co. ("Lilly")*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997), the Examiner states that

> the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show that Applicant was in possession of

> the claimed genus.
> *4 ....
> In the instant case, *there is no structural limitation* recited with regard to *all* the members of the genus of polynucleotides recited.

(Answer 5.)

In our opinion, *Lilly* is not the proper standard to apply to the claims in this appeal. In *Lilly*, at issue was the written description of a novel DNA genus. *Lilly*, 119 F.3d at 1563, 1567, 43 USPQ2d at 1401, 1405. In this case, DNA sequences for the claimed enzymes were known in the prior art. It is unnecessary for a patent application to provide a description of nucleotide sequences which are already known in the prior art. *Falko-Gunter Falkner v. Inglis*, 448 F.3d 1357, 1367, 79 USPQ2d 1001, 1008 (Fed. Cir. 2006). As explained in *Capon v. Eshhar*, 418 F.3d 1349, 1358, 76 USPQ2d 1078, 1084-5 (Fed. Cir. 2005):

> The "written description" requirement must be applied in the context of the particular invention and the state of the knowledge. The Board's rule that the nucleotide sequences of the chimeric genes must be fully presented, although the nucleotide sequences of the component DNA are known, is an inappropriate generalization. When the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined afresh. Both parties state that a person experienced in the field of this invention would know that these known DNA segments would retain their DNA sequences when linked by known methods. Both parties explain that their invention is not in discovering which DNA segments are related to the immune response, for that is in the prior art, but in the novel combination of the DNA segments to achieve a novel result.

Here, the claimed enzymes - hydantoinase, hydantoin racemase, and D- or L-carbamoylase - were well-known and characterized in the prior art. As explained by Appellants, enzyme activities for each of the three enzyme classes were known in the art prior to the filing date of the application (Br. 13). Conserved amino acid motifs had been established for each enzyme class (Br. 16, 18, 20 (Tables 1-3)). Nucleotide information for these enzymes was also known and could be deduced from the amino acid sequences based on the known genetic code (Br. 21). Like the circumstances in *Capon*, Appellants are not claiming to have discovered the DNAs recited in claim 17; they are prepared from known DNA sequences of known function. The Examiner erred in concluding that the Specification does not meet the written description requirement because it does not reiterate the structure of the claimed genus of known enzymes. We reverse the rejection of claims 17-19, 21-24, 26-28, 30-38, 42, 45, and 46 for lack of written description.

Rejection under § 112, first paragraph for lack of enablement

*5 Claims 17-19, 21-24, 26-28, 30-38, 42, 45, and 46 stand rejected under § 112, first paragraph, for lack of enablement (Answer 8). The Examiner states that it would require undue experimentation to practice the claimed invention with "a microorganism transformed with a DNA encoding any hydantoinase, hydantoin racemase and/or carbamoylase" (Answer 8). The Examiner asserts "[t]he scope of the claims ... is not commensurate with the enablement provided in regard to the extremely large number of unknown DNAs encoding any hydantoinase, hydantoin racemase, or carbamoylase required to practice the claimed invention" (Answer 8). The Examiner contends that the example of enzymes from one strain of *Arthobacter* (SEQ ID NOS: 8, 10, and 6) is not sufficient to enable the full scope of the claim because there is no information about the structure of other hydantoinases, hydantoin racemases, and carbamoylases (Answer 9). The Examiner also states that it would not be routine "to isolate/create any polynucleotide encoding a protein with the activity recited without any knowledge as to the structural features which would correlate with that activity" (Answer 9).

"To be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" *Genentech, Inc. v. Novo Nordisk, A/S*, 108 F.3d 1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997). The Examiner's rejection is based on the breadth of the claimed genus in covering enzyme coding sequences which are not disclosed or described in the Specification. However, Appellants have provided evidence that the claimed enzymes - hydantoinase, hydantoin racemase and D- or L-specific carbamoylases - had been characterized in the prior art and that many examples of each enzyme type were known prior to the filing date of the application (Br. 12-21).

Appellants also present evidence that conserved amino acid motifs involved in enzyme catalysis were known for each enzyme class (Br. 14-20). While the Examiner acknowledges the existence of these conserved motifs, the Examiner contends that "it is unlikely that these small motifs [are] all that is required for a protein to have the recited enzymatic activity since the catalytic sites for enzymes are expected to be larger than 5 amino acids" (Answer 26). We do not find this persuasive. First, the catalytic regions of each enzyme class are not characterized as having less than 5 amino acids. For hydantoinases, conserved residues span almost 200 amino acids (e.g., from 56-239) (Br. 16); for hydantoinase racemase, more than nine amino acids (e.g., from 196-208;

from 196-204) (Br. 18). Secondly, because a large number of enzymes were known in the prior, including their identifying and functional characteristics, this would have aided the skilled person in the construction of additional enzymes within the claim scope.

*6 We also find Appellant' arguments persuasive that it would not require undue experimentation to express the genus of claimed enzymes in bacteria, and use the bacteria to produce amino acids, because such methods were well known in the art at the time the application was filed. (Br. 24-26).

In sum, we conclude that a person of ordinary skill in the art would have clearly possessed sufficient knowledge to make and use the full scope of the claims. We reverse the rejection of claims 17-19, 21-24, 26-28, 30-38, 42, 45, and 46 as lacking enablement.

Rejection under § 103

Claims 17, 18, and 30 stand rejected under 35 U.S.C. § 103(a) as obvious over Wagner (Answer 10).

Wagner teaches microorganisms which are capable of converting 5-monosubstituted hydantoins or N-carbamoyl alpha amino acids into pure L-amino acids using a carbamoylase, hydantoinase, and hydantoin racemase (Wagner, col. 1, ll. 8-12; Answer 10). Wagner also describes obtaining a gene coding for a carbamoylase, hydantoinase, or hydantoin racemase (Wagner, col. 1, ll. 65-67). "As persons of ordinary skill would appreciate, these genes may be useful for" inserting into a microorganism to "produce large amounts of the enzyme(s)" (Wagner, col. 3, ll. 28-36).

In reaching an obviousness determination, it is necessary to identify the differences between the claimed invention and the prior art, and then to determine whether these differences are obvious in view of the scope and content of the prior art and the level of skill in the pertinent art. *Graham v. John Deere Co.*, 383 U.S. 1, 13-14, 148 USPQ 459, 465 (1966). The Examiner finds that Wagner teaches inserting a gene coding for a carbamoylase, hydantoinase, and/or hydantoin racemase, but does not teach "a microorganism transformed with a plasmid containing DNA encoding a carbamoylase, hydantoinase, and a hydantoin racemase, wherein said DNAs are expressed at rates which result in reduced accumulation of intermediates in the conversion of 5-monosubstituted hydantoins to L- or D-amino acids" as required by claim 17 (Answer 10). However, the Examiner concludes that the claimed expression method would have been obvious because

> D- and L- amino acids are widely used biochemicals, therefore methods of making such amino acids are highly desirable. Also, one of skill in the art is motivated to express these genes at rates which would avoid accumulation of intermediates because accumulation of intermediates can potentially reduce yield and is not efficient.

(Answer 11).

The Examiner bears the initial burden of showing unpatentability. *See, e.g., In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). "[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006) (quoted in *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct 1727, ___, 82 USPQ2d 1385, 1396 (2007)). Common knowledge and common sense are a part of this reasoning. *See DyStar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co.*, 464 F.3d 1356, 1367, 80 USPQ2d 1641, 1650 (Fed. Cir. 2006). However, in this case, the Examiner has only made conclusory statements, and has not presented sufficient evidence that a person of ordinary skill in the art would have known to overexpress hydantoinase, hydantoin racemase, and carbomoylase according to their turnover rates in order "to reduce the accumulation of intermediates in the conversion of the 5-monosubstituted hydantoins to L- or D-amino acids" as required by claim 17.

*7 Wagner describes gene cloning and expression of cloned genes coding for amino acid producing enzymes (Wagner, col. 3, ll. 25-40), but does not disclose specific cloning or expression methods. Wagner also states that a "person of ordinary skill would appreciate" that the cloned genes could be used for expression purposes (Wagner, col. 3, ll. 28-32). Based on this evidence, it is reasonable to presume that expression methods were well-known in the art and that one of ordinary skill did not require explicit instructions on how to express genes.

Although the skilled worker was knowledgeable about cloning and expressing genes, and may have had reason to express all *three* genes in a single microorganism, the Examiner presents no evidence that the skilled worker would have known to overexpress the genes "according to the[ir] turnover rates ... to reduce the accumulation of intermediates in the conversion of the 5-monosubstituted hydantoins to L- or D-amino acids" as required by claim 17. The Examiner states that "accumulation of intermediates can potentially reduce yield and is not efficient" (Answer 11), but provides no support that this was common knowledge in this field.

Fig. 6 of the Specification shows that not all methods in

which the three genes are co-expressed in a microorganism result in reducing intermediate accumulation (*see supra* on p. 5). In sum, there is nothing in the record that would lead us to believe that adjusting the co-expression levels according to "turnover rates" to reduce intermediate accumulation is an obvious solution to the problem of amino acid production addressed by Appellants' claims.

For the foregoing reasons, we reverse the rejections of claims 17, 18, and 30 as obvious over Wagner.

Rejection under nonstatutory obvious-type double-patenting

Claims 17-19, 21-24, 26-28, 30-38, and 41-46 stand rejected on the ground of nonstatutory obvious-type double patenting over claims 1-56 of US 6,713,288 (Answer 12).

Appellants request that the rejection "be held in abeyance until the time allowable subject matter is identified" (Br. 27). Since Appellants have not disputed the merits of the rejection, we affirm it.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a)(1)(iv)(2006).

AFFIRMED

FN1. "Turnover rate" refers to the number of substrate molecules in a certain time period that an enzyme can process. Bruce Alberts, *Molecular Biology of the Cell* 163 (4th Edition, 2002).

2007 WL 1766992 (Bd.Pat.App. & Interf.)
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